# Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US05/005554

International filing date: 22 February 2005 (22.02.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/546,436
Filing date: 20 February 2004 (20.02.2004)

Date of receipt at the International Bureau: 07 April 2005 (07.04.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

1302309

# THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

*March 31, 2005*

**THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.**

**APPLICATION NUMBER:** *60/546,436*
**FILING DATE:** *February 20, 2004*
**RELATED PCT APPLICATION NUMBER:** *PCT/US05/05554*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

PTO/SB/16 (08-03)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it display a valid OMB control number



# *PROVISIONAL APPLICATION FOR PATENT COVER SHEET*

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

**Express Mail Label No.** **EU817303121US**

**INVENTOR(s)**

| Given Name (first and middle [if any]) | Family Name or Surname | Residence (City and either State or Foreign Country) |
|---|---|---|
| Elliot L. | Chaikof | Atlanta, Georgia 30350 |
| Chrystelle S. | Cazalis | Atlanta, Georgia 30329 |
| Carolyn A. | Haller | Atlanta, Georgia 30328 |

**TITLE OF THE INVENTION (500 characters max)**

Thrombomodulin Conjugates

*Direct all correspondence to:* **CORRESPONDENCE ADDRESS**

☐ Customer Number _____ *Type Customer Number here*

*Place Customer Number Bar Code Label Here*

*OR*

| | | | | | |
|---|---|---|---|---|---|
| ☒ Firm *or* Individual Name | Office of Technology Transfer | | | | |
| *Address* | Emory University | | | | |
| *Address* | 1784 N. Decatur Rd., Suite 130 | | | | |
| *City* | Atlanta | *State* | GA | *Zip* | 30322 |
| *Country* | United States | *Telephone* | 404-727-2211 | *Fax* | 404-727-1271 |

**ENCLOSED APPLICATION PARTS** *(check all that apply)*

☒ Specification *Number of Pages* 21 ☐ CD(s), Number _____

☒ Drawing(s) *Number of Sheets* 7 ☐ Other (specify) _____

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees

☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 50-13-11

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☒ Yes, the name of the U.S. Government agency and the Government contract number are: NIH RO1HL56819

*Respectfully Submitted,*

SIGNATURE Todd Sherer

Date 02/20/04

TYPED OR PRINTED NAME Todd T. Sherer

REGISTRATION NO. *(if appropriate)* 39,369

TELEPHONE 404-727-5256

Docket Number: 04027Prov

The PTO did not receive the following listed item(s) One page of drawings

## USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the PTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademenark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*Express Mail No: EU817303121US*

EMORY UNIVERSITY

Office of Technology Transfer

Mail Stop Provisional Patent Application
Commissioner of Patents and Trademarks
P.O. Box 1450
Alexandria, VA 22313-1450

**Re: U.S. Provisional Patent Application**
**Filed: February 20, 2004**
**For: "THROMBOMODULIN CONJUGATES"**
**Emory File No.: 04027Prov**

Dear Sir:

Enclosed for filing regarding the above-referenced patent application are:

(1) PROVISIONAL APPLICATION COVER SHEET
(2) SPECIFICATION AND DRAWING
(3) SMALL ENTITY STATEMENT
(4) AUTHORIZATION TO PAY FROM DEPOSIT ACCOUNT IN THE AMOUNT OF $80.00; AND
(5) RETURN POSTAL CARD.

The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-13-11.

Sincerely,

Todd T. Sherer, Ph.D.
Reg. No. 39,369

Enclosures

**CERTIFICATE OF EXPRESS MAILING**

I hereby certify that this correspondence is being deposited with the United States Postal Service as **Express Mail No EU817303121US** in an envelope addressed to:

Mail Stop Provisional Patent Application
Commissioner of Patents and Trademarks
P.O. Box 1450
Alexandria, VA 22313-1450

on this 20th day of February, 2004.

Orman A. Simpson — 2/20/04 (Date)

Emory University
1784 North Decatur Road, Suite 130
Atlanta, Georgia 30322
*An equal opportunity, affirmative action university*

Tel 404.727.2211
Fax 404.727.1271

When completed submit to:

Office of Technology Transfer
1794 North Decatur Road, Suite 130
Atlanta, Georgia 30322
(404) 727-2211

# Emory Intellectual Property Disclosure Statement Form

**Confidential Information**

( for OTT internal use only)
File No. _____
Publication Yes ☐ No ☐
Government Yes ☐ No ☐
OTT Reviewer _____

The purpose of this form is to facilitate the prompt disclosure by Emory Personnel of any Intellectual Property to which Emory may assert ownership rights pursuant to Section I of the Emory University Intellectual Property Policy (adopted June 6, 2002).
Please review the attached instructions and guidelines.

1. Title of Intellectual Property Thrombomodulin Conjugates

2. Type of Intellectual Property

| | | | | | |
|---|---|---|---|---|---|
| Biological Material | ☑ | Computer Software | ☐ | Medical Device | ☐ |
| Method of Synthesis | ☐ | Therapeutic Materials | ☑ | Other | ☐ |

3. Please attach a description of the Intellectual Property

4. Contributor(s)

| Position | Department | Campus Address & Phone | Email Address |
|---|---|---|---|
| Elliot L. Chaikof, MD, PhD | Professor | Surgery | 1639 Pierce Drive, Rm 5105, 7-8409 | echaiko@emory.edu |
| Chrystelle S. Cazalis, PhD | Research Associate | Surgery | as above | |
| Carolyn A. Haller, PhD | Sr. Research Technician | Surgery | as above | |

(Please list all persons who are believed to have made significant contributions to the Intellectual Property including Emory Personnel as well as other Contributors. Determination of inventorship is a legal matter and will be determined by legal counsel.)

5. Was this intellectual property developed with the use of any research grant/contract funds? Yes ☑ No ☐

| Contract No(s) | Sponsor(s) | OSP UPN No(s) | Principal Investigator |
|---|---|---|---|
| RO1HL56819 | NIH | | Elliot L. Chaikof, MD, PhD |

6. Please list any Material Transfer Agreements (incoming and/or outgoing) or other agreements (other than those listed in 5 above) that are related to this Intellectual Property

| Institution/Company | OTT No(s) | Date(s) | Material |
|---|---|---|---|

| 7. **Public Disclosure** (accurate data is essential as prior disclosure may affect the possibility of obtaining patent rights) | Date | References/Comments Please include names of periodicals/journals (use separate sheet if necessary) |
|---|---|---|
| A. First publication containing sufficient description to enable a person skilled in this field to understand and to make or use the Intellectual Property (include these, and the date submitted) | | |
| B. First public oral disclosure of Intellectual Property sufficient to enable a person skilled in this field to understand and to make or use the invention (include slides and/or presentation material) | | |
| C. If this Intellectual Property is unpublished and undisclosed, provide the anticipated publication or oral disclosure date and any submissions made for potential publication. | | |
| D. Other publications and/or public oral disclosures that provide background information to this Intellectual Property | | |

## Confidential Information

8. Has the Intellectual Property been reduced to practice? Yes ☐ No ☐

9. Please attach list of any commercial entities that may be interested in this Intellectual Property (provide as much detail as possible)

10. Please list three Emory reviewers who are knowledgeable in the area of this Intellectual Property.

| Name | Department | Campus Address/email |
|---|---|---|
| 1. | | |
| 2. | | |
| 3. | | |

11. This Intellectual Property was disclosed to and understood by:

Signature of Non-Inventor Emory Personnel Witness _____ Date _____

Name and Title of Witness (please type or print) _____

**I (we) hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.**

**I (We) hereby agree to assign all right, title and interest to this Intellectual Property to Emory University and agree to execute all documents as requested, assigning to Emory University our rights to any patent application filed on this Intellectual Property, and to cooperate with the Emory University Office of Technology Transfer in the protection of this Intellectual Property. Emory University will share any royalty income derived from the Intellectual Property with the inventor(s) according to its standard policies.**

| | |
|---|---|
| [signature] 11/6/03 | Chrystelle CAZALIS 11/7/03 |
| Contributor's Signature (Emory Personnel) Date | Contributor's Signature (Emory Personnel) Date |
| 150 Wildwood Glen Atlanta | 3078 Clairmont road, #433 ATLANTA, GA 30329 |
| Home Address City, State, Zip | Home Address City, State Zip |
| 226 88 4324 30350 USA | 670-07-0073 FRANCE |
| Social Security No. (required) Country of Citizenship | Social Security No. (required) Country of Citizenship |

| | |
|---|---|
| Carolyn A Hall 11/7/03 | |
| Contributor's Signature (Emory Personnel) Date | Contributor's Signature (Emory Personnel) Date |
| 6851 Roswell Rd #H28 Atlanta GA 30328 | |
| Home Address City, State, Zip | Home Address City, State, Zip |
| 294-84-7324 USA | |
| Social Security No. (required) Country of Citizenship | Social Security No. (required) Country of Citizenship |

Please note that Social Security number and country of citizenship are required; absence of this information may hinder distribution of the inventor's share of any royalties that may result from this technology. If there are more than four Emory Contributors, please attach an additional form.

Express Mail No.: EU817303121US

# C-Terminal Site-Specific PEGylation of a Truncated Thrombomodulin with Full Bioactivity

Chrystelle S. Cazalis, Carolyn A. Haller and Elliot L. Chaikof†

Department of Surgery
Emory University
1639 Pierce Drive
Atlanta, GA 30322

General purpose

i. **Technical description, including a list of key words**

The present invention is focused on the generation of novel soluble thrombomodulin ("TM") conjugates, which we believe will be useful as a systemic agent for treatment of micro or macrovascular blood clots, stroke, heart attack, disseminated intravascular coagulation or other inflammatory or prothrombotic condition. The sequence of the TM analogs contains, at a minimum, the catalytically active site capable of activating protein C (EGF4-6 domains) and single or multiple non-natural amino acids. These TM analogs are conjugated to linear or branched natural or synthetic polymers via the non-natural amino acids. This invention provides methods for conjugation of the TM conjugates to the surfaces of synthetic or natural materials, to targeting groups for site specific delivery of the agent, or to compounds that contain one or more additional anti-inflammatory/anti-thrombotic properties.

Keywords: thrombomodulin, catalytically active sites, non-natural amino acid, conjugation, natural polymer, synthetic polymer, poly(ethylele glycol), surface anchoring, anti-inflammatory, anti-thrombotic.

ii. **Advantages, unusual features and improvements over existing methods, devices or materials. How does the Intellectual Property differ from the present technology? What problems does it solve or what advantages does it possess?**

The transmembrane human protein thrombomodulin ("TM"), as a critical regulator of the protein C pathway, represents the major anticoagulant mechanism that is operative in both normal and injured blood vessels under physiologic conditions in vivo. An effective blood-contacting surface is dependent upon the presence of physiologically relevant antithrombogenic mechanisms that are incorporated into the engineered blood-material interface. Full length TM can be incorporated into membrane-mimetic film or surfaces by fusion/adsorption processes. The major drawback of these materials is a loss of TM stability with time. The invention **solves** this problem by a covalent conjugation of truncated TM derivatives onto the surface using natural or synthetic polymers as a spacer. Moreover, in existing protocols for covalent immobilization of TM onto polymeric surfaces, the protein immobilization procedure involves freely available amino or carboxyl functionalities of TM, some of which may be within or near a bioactive site. Therefore, TM bioactivity is significantly reduced after surface coupling. The invention provides a **novel method** for covalent conjugation of TM to synthetic or natural materials site-specifically without loss of protein bioactivity. The invention is based on a short recombinant TM construct containing EGF-like domains 4-6 and single or multiple non-natural amino acids preferably at the C-terminal portion of the construct. Through reaction with suitable polymer spacer, via the non-natural amino acids TM was modified for further immobilization onto surfaces or for conjugation to linear or multifunctional natural or synthetic compounds that contain other anti-inflammatory or anti-thrombotic properties. The bioconjugation reactions occur in mild conditions to preserve TM

**iv. Commercial applications (economic potential, potential uses, indirectly related uses, etc.)**

The TM conjugates can be used as systemic agents for treatment of micro or macrovascular blood clots, stroke, heart attack, disseminated intravascular coagulation or other inflammatory or prothrombotic condition. An embodiment of the invention consists of coating of the surface of medically implanted or human tissue or fluid contacting devices including but not restricted to vascular grafts, stents, heart valves, dialysis membranes, membrane oxygenators, catheters, or guide wires to alter surface properties. A further embodiment of the invention consists of coating of living cells or tissues, including, but not restricted to smooth muscle cells, fibroblasts, endothelial cells, stem cells, chondrocytes, osteoblasts, pancreatic islets, or genetically engineered cells to enhance the anti-inflammatory properties of the cells.

v. **Please list (and attach) any additional related patents or publications, which may be important background reference material in reviewing the Intellectual Property. If appropriate, please attach an initial patent search related to the Intellectual Property obtained from searching the USPTO's website: http:www.uspto.gov/web/menu/search.html**

Patents:

| | |
|---|---|
| 5,126,140 | Thrombomodulin-coated biocompatible substance |
| 5,834,028 | Soluble thrombomodulin-containing composition |
| 5,863,760 | Protease-resistant thrombomodulin analogs |
| 5,583,102 | Human thrombomodulin in wound healing |
| 6,632,791 | Thrombomodulin analogs for pharmaceutical use |
| 5,256,770 | Oxidation resistant thrombomodulin analogs |
| 5,108,759 | Endothelial envelopment drug carriers |
| 6,410,057 | Biodegradable mixed polymeric micelles for drug delivery |

References


- Weiler, H., Isermann, B. H. Thrombomodulin. (2003) *J. Thrombosis and Haemostasis 1,* 1515-1524.
- Kalafatis, M., Egan, J.O., Van't Veer, C., Cawthern, K. M., Mann, K. G. (1997) The regulation of clotting factors. *Crit. Rev. Eukaryot. Gene Expr. 7*, 241-280.
- Esmon, N. L., Owen, W.G., Esmon, C. T. (1982) Isolation of a membrane-boud cofactor for thrombin-catalyzed activation of protein-C. *J. Biol. Chem. 257*, 859-864.
- Esmon, C. T. (1992) The regulation of clotting factors. A*rteriosclevoris and Thrombosis 12*, 135-145.
- Parkinson, J. F., Nagashima, M. M, Kuhn, I., Leonard, J., Morser, J. (1992) Structure-Function studies of the epidermal growth-factor domains of human thrombomodulin. *Biochem Biophys. Res. Commun. 185*, 567-576.
- Clarke, J. H., Light, D. R., Blasko, E. J., Parkinson, F., Nagashima, M., McLean, K., Vilander, L., Andrews, W. H., Morser, J., Glaser, C. B. (1993) The short loop

between epidermal growth factor-like domain-4 and domain-5 is critical for human thrombomodulin function. *J. Biol. Chem. 268*, 6309-6315.

- Nagashima, M., Lundh, E., Leonard, J. C., Morser, J., Parkinson J. F. (1993) Alanine-scanning mutagenesis of the epidermal growth factor-like domains of human thrombomodulin identifies critical residues for its cofactor activity. *J. Biol. Chem. 268*, 2888-2892.
- Glaser, C. B., Morser, J.,Clarke, J. H., blasko, E., McLean, K., Kuhn, I., Chang, R. –J., Lin, J.-H., Vilander, L., Andrews, W. H., and Light, D. R. (1992) Oxidation of a secific methionine in thrombomodulin by activated neutrophil products blocks cofactor activity-a potential rapid mechanism for modulation of coagulation. *Clin. Invest. 90*, 2565-2573.
- Kiick, K. L., Saxon, E., Tirrell, D. A., Bertozzi, C.R. (2002) Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. *PNAS 99*, 19-24.
- Saxon, E., Bertozzi, C. R. (2000) Cell surface engineering by a modified Staudinger reaction. *Science 287*, 2007-2010.
- Veronese, F. M. (2001) Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials 22*, 405-417.
- Roberts, M. J., Bentley, M. D., Harris, J. M. (2002) Chemistry for peptide and protein PEGylation. *Adv Drug delivery reviews 54*, 459-476.
- Katre, N. V. (1993) The conjugation of proteins with polyethylene glycol and other polymers. Altering properties of proteins to enhance their therapeutic potential. *Adv Drug Delivery Rev 10,* 91–114.
- Monkarsh, S. P., Ma, Y. M., Aglione, A., Bailon, P., Ciolek, D., DeBarbieri, B., Graves, M. C., Hollfelder, K., Michel, H., Palleroni, A., Porter, J. E., Russoman, E., Roy, S., Pan, Y. C. E. (1997) Positional isomers of monopegylated interferon α2a: isolation, characterization, and biological activity. *Anal. Biochem. 247*, 434-440.
- Han, H. -S., Yang, S. -L., Yeh, H. -Y., Lin, J. -C., Wu, H. -L., Shi, G. -Y. (2001) Studies of a novel human thrombomodulin immobilized substrate: surface characterization and anticoagulation activity evaluation. *J. Biomater. Sci. Polymer edn, 12*, 1075-1089.
- Tsutsumi, Y., Onda, M., Nagata, S., Lee, B., Kreitman, R. J., Pastan, I. (2000) Site-specific chemical modification with polyethylene glycol of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) improves antitumor activity and reduces animal toxicity and immunogenicity. *Proc. Natl. Acad. Sci. USA 97*, 8548-8553.
- He, X. H., Shaw, P. C., Xu, L. H., Tam, S. C. (1999) Site-directed polyethylene glycol modification of trichosanthin: effects on its biological activities, pharmacokinetics, and antigenicity. *Life Sci. 64*, 1163-1175.
- Goodson, R. J., Katre, N. V. (1990) Site-directed pegylation of recombinant interleukin-2 at its glycosylation site. *Biotechnology 8*, 343–346.
- Kogan, T.P. (1992) The synthesis of substituted methoxy-poly(ethylene glycol) derivatives suitable for selective protein modification. *Synth. Commun. 22*, 2417–2424.
- Morpurgo, M., Veronese, F. M., Kachensky, D., Harris, J. M. (1996) Preparation and characterization of poly(ethylene glycol) vinyl sulfone. *Bioconjug. Chem. 7*, 363–368.

- Woghiren, C., Sharma, B., Stein, S. (1993) Protected thiol-polyethylene glycol: a new activated polymer for reversible protein modification. *Bioconjug. Chem. 4*, 314–318.
- Karpusas, M., Nolte, M., Benton, C. B., Meier, W., Lipscomb, W. N., Goelz, S. (1997) The crystal structure of human interferon beta at 2.2-A resolution. *Proc. Natl. Acad. Sci. USA 94*, 11813–11818.
- Kuan, C. T., Wang, Q. C., Pastan, I. (1994) *Pseudomonas* exotoxin A mutants. Replacement of surface-exposed residues in domain II with cysteine residues that can be modified with polyethylene glycol in a site-specific manner. *J. Biol. Chem. 269*, 7610-7616.
- Yamamoto, Y., Tsutsumi, Y., Yoshioka, Y., Nishibata, T., Kobayashi, K., Okamoto, T., Mukai, Y., Shimizu, T., Nakagawa, S., Nagata, S., Mayumi, T. (2003) Site-specific PEGylation of a lysine-deficient TNF-alpha with full bioactivity. *Nature Biotechnology 21*, 546-552.
- Kurfurst, M. M. (1992) Detection and molecular weight determination of polyethylene glyol-modified hirudin by staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem. 200*, 244-248.
- Manjula, B. N., Tsai, A., Upadhya, R., Perumalsamy, K., Smith, P. K., Malavalli, A., Vandegriff, K., Winslow, R. M., Intaglietta, M., Prabhakaran, M., Friedman, J. M., Acharya, A. S. (2003) Site-specific PEGylation of hemoglobin at cys-93(beta): Correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain. *Bioconjugate Chemistry 14*, 464-472.
- Feng, J., Tseng, P. -Y., Faucher, K. M., Orban, J. M., Sun, X. -L., Chaikof, E. L. (2002) Functional reconstitution of thrombomodulin within a substrate-supported membrane-mimetic polymer film. *Langmuir 18*, 9907-9913.
- Kishida, A., Ueno, Y., Maruyama, I., Akashi, M. (1994) Immobilization of human thrombomodulin on biomaterials-Evaluation of the activity of immobilized human thrombomodulin. *Biomaterials 15*, 1170-1174.
- Kishida, A., Ueno, Y., Maruyama, I., Akashi, M. (1994) Immobilization of human thrombomodulin on onto poly(ether urethane urea) for developing antithrombogenic blood-contacting materials. *Biomaterials 15*, 848-852.

# ABSTRACT

Addition of polyethylene glycol to bioactive proteins (PEGylation) improves their plasma half-life and stability against proteolytic cleavage and may also decrease protein immunogenicity. Characteristically, PEGylation usually involves the reaction to available lysine amino groups, some of which may be within or near a bioactive site. Thus, most protocols are nonspecific and result in a loss of protein activity. We report herein a strategy for site-specific PEGylation of a thrombomodulin derivative (TM) at the C-terminus. A truncated TM mutant consisting of epidermal growth factor (EGF)-like domains 4-6 with a C-terminal azido-alanine was expressed in *Escherichia coli*. The TM mutant was site-specifically conjugated to a methyl-PEG-triarylphosphine compound *via* Staudinger reaction. .Enzymatic activity of the TM construct before and after PEGylation was similar, which demonstrated the success and usefulness of our novel site-specific PEGylation approach.

# INTRODUCTION

Pegylation of proteins increases both their molecular size and steric hindrance, which results in an improvement of protein plasma half-life and resistance to proteolytic cleavage. In addition, protein immunogenicity may be decreased (*1,2*). Characteristically, PEGylation usually involves the reaction to available lysine amino groups, some of which may be within or near a bioactive site. Thus, most protocols are nonspecific and result in a loss of protein activity (*3,4*). For example, Han *et al.* (*5*) conjugated TM to PEG via trichlrotriazine as the coupling agent for immobilization onto glass surface. Despite successful immobilization, they reported reduced TM activity presumably due to the alteration of protein conformation after PEGylation. To overcome this problem, several approaches have been proposed. Site-specific PEGylation can be achieved through the introduction of a cysteine residue in engineered proteins with a free thiol available for conjugation reactions (*6,7*). Several PEG derivatives have been developed for that purpose (*8,9,10,11,12*). The efficacy of this approach is compromised, however, by a low yield of PEGylated proteins and often by a substantial loss of activity due to the introduction of the cysteine residue (*13*). As an alternative strategy, Yamamoto *et al.* (*14*) reported the site-specific PEGylation of a lysine-deficient tumor necrosis factor-α at its N-terminus using an activated PEG (metoxy-PEG-succinimidyl propionate). The corresponding bioconjugate showed higher bioactivity *in vitro* and greater antitumor therapeutic potency than randomly mono-PEGylated TNF-α.

We report herein the use of a similar strategy for site-specific PEGylation of human thrombomodulin. This transmembrane protein is a critical regulator of the protein C pathway and represents a major anticoagulant mechanism that is operative under physiologic conditions *in vivo* (*15,16*). TM is a cofactor for thrombin-catalyzed activation of protein C, enhancing the rate of the reaction by 1000-fold (*17*). In order to closely mimic the TM structure as it appears at the cell surface (*18*), and consequently preserve its bioactivity, we investigated the PEGylation at the C terminus. Using a genetically-directed synthesis in *Escherichia coli*, we first expressed a short TM construct containing EGF-like domains 4-6 and an azido-functionalized methionine analog (*19*) as C-terminal linker. The PEGylation was then achieved through Staudinger ligation (*20*) with a suitable engineered PEG derivative.

# EXPERIMENTAL PROCEDURES

**Materials.** All chemical reagents were obtained from Sigma Chemical Corporation (St. Louis, MS). The PEG analog was purchased from Netkar Corp. (Hunstville, AL). The BamH I and Shrimp Alkaline Phosphatase enzymes were obtained from New England Biolabs, Inc. (Beverly, MA). The Quikchange Site-Directed Mutagenesis kit was from Stratagene (La Jolla, CA). *E.coli* strain B834 (DE3), plasmid pET-39b(+), S-Tag Rapid Assay kit and Site-Specific *Enterokinase* Cleavage and Capture Kits were from Novagen (Madison, WI). All plasmid purification kits were purchased from QIAGEN Inc. (Chatsworth, CA). The mouse monoclonal antibody to human thrombomodulin was from COVANCE Corp. (Richmond, CA). Synthetic oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville,

IA). Purified recombinant human PC and Rabbit Lung Thrombomodulin and were from Haematologic Technologies Inc. (Essex Junction, VT). Human anti-trombin, recombinant Human Thrombomodulin positive control and chromogenic substrate SPECTROZYME PCa were purchased from Amercian Diagnostica Inc. (Stamford, CT). All reagents for manipulating DNA and bacteria were sterilized by autoclave.

**Instrumentation.** MALDI-TOF mass spectrometry data were performed on an Applied Biosystem Voyager-DE™ STR Biospectrometry™ Workstation MALDI-TOF Mass Spectrometer using an 2-(4-hydroxy-phenylazo)benzoic acid matrix. $^{1}H$, $^{13}C$ and $^{31}P$ NMR spectra were recorded at 400, 150 and 242 MHz on a Varian INOVA in $CDCl_3$ or $D_2O$ (internal $Me_4Si$, $\delta$=0). Optical density was recorded on a Varian Cary 50 Bio UV-visible spectrophotometer.

**Synthetic Gene Construction.** A DNA fragment encoding for EGF (4-6) domains of human TM was obtained by polymerase chain reaction using primers 5'-TACCCTAACTACGACCTGGTG-3' and 5'-TATGAGCAAGCCCGAATG-3'. Through a series of intermediate constructs, this fragment was used to generate a gene containing a Leucine (Leu) substitution for Methionine-388 (Met-388), N-terminal and C-terminal BamH I sites and a C-terminal linker GlyGlyMet using site-directed mutagenesis. The final construct ($\mathbf{TM_{GGM}}$) was then inserted using the BamH I site of the expression plasmid pET$_T$39b(+). All mutations were verified through sequence analysis.

**Protein expression and purification.** M9AA medium (500 ml) supplemented with 1 mM MgSO4, 0.4 wt% glucose, 1 mg/liter thiamine chloride, 0.1 mM $CaCl_2$, the Kanamycin antibiotic (30 mg/liter) and all proteinogenic amino acids (40mg/L) was inoculated with 20 ml of an overnight culture of B834(DE3)/pET39-b/$\mathit{TM_{GGM}}$. When the turbidity of the culture reached an $OD_{600}$ of 0.8, protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. After 10min, the medium was exchanged to remove methionine: the cells were sedimented (4000g, 10min) then the cell pellet was washed twice with 200 ml of 1x M9 salts. The cells were resuspended in 500 ml of the M9AA medium described above, without methionine but supplemented with 100 mg/liter of azido-functionalized methionine analog (*19*). A culture lacking methionine served as the negative control. Cultures were grown for 4.5 h at 37°C. The expression of the TM protein was analyzed by 4-20% gradient SDS-PAGE gel electrophoresis and visualized by Western blot analysis using mouse monoclonal antibody to human thrombomodulin. The target protein was expressed as a N-terminal Dsba and Enterokinase enzymes fusion to a leader sequence containing hexahistine and S-tags (protein $\mathbf{TM_A}$). S-Tag Rapid Assay was used to quantify protein concentration and therefore expression yield, which can be averaged as 17mg per liter of cell culture. $\mathbf{TM_A}$ was purified from the cell pellet by using immobilized metal-affinity chromatography on TALON resin (Clontech Laboratories, Inc.) under native conditions using an imidazole gradient for elution of the target polypeptide. The cells were first harvested by centrifugation at 4 ℃ at 4000 *g* for 15 min, and resuspended in 4 mL of lysis buffer (300 mM NaCl, 50 mM $NaH_2PO_4$, 10% glycerol, 1 mg/ml lysozyme, 10μg/ml PMSF, pH 8). After incubation on ice for 30min, the cell lysate was clarified by centrifugation at 10000*g* for 20 min. The soluble extract was then loaded onto a column containing TALON metal affinity resin (4 mL), which had been preequilibrated with lysis buffer. The weakly binding proteins were removed

by rinsing the column with 16 mL wash buffer (300 mM NaCl, 50 mM $NaH_2PO_4$, 10% glycerol, 20mM imidazole, pH 8). $TM_A$ was eluted by the addition of 6 mL of elution buffer (300 mM NaCl, 50 mM $NaH_2PO_4$, 10% glycerol, 250mM imidazole, pH 8). The chromatographic fractions were analyzed by 4-20% gradient SDS-PAGE gel electrophoresis and visualized by Western blot analysis using mouse monoclonal antibody to human thrombomodulin. The nitrocellulose membrane was developed by using ECL plus Western Blotting detection kit (Amersham Biosciences, UK). *Enterokinase* cleavage removed the fusion tag and generated the target protein ($TM_B$). N-terminal sequencing, amino acid compositional and mass analysis confirmed the integrity of $TM_B$: (Mass detected (m/z): 16 545.2 D (calculated 16 540.0 D)).

**Activity assays.** A saturated concentration of $TM_B$ was incubated in assay diluents of 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 2.5 mM $CaCl_2$ for 30 min at 37°C in 20μl total volume with various amount of protein C (0-8 μM) and 10 nM thrombin. The reaction was stopped by addition of 5 μL antithrombnin (10.7 μM) for 5 min at room temperature. The amount of protein C formed was determined by addition of 275μL of SPECTROZYME PCa (218 μM) for 20 min at room temperature and monitoring its hydrolysis at 407 nm using UV spectrophotometer. The activated protein C activity values obtained in the absence of TM were subtracted to give to final values.

**Synthesis of the methyl-PEG-triarylphosphine conjugate.** To a mixture of the phospino reagent (10.3 mg, 28 μmol) and 1,3-Dicyclohexylcarbodiimide (DCC) (5.7 mg, 27 μmol) in $CHCl_3$ (2 ml) was agitated for 1h at room temperature under Argon. Then a solution of the methyl-PEG-amine derivative (m-PEG-$NH_2$ ($M_n$ 5kD)) (120 mg, 24 μmol) in $CHCl_3$ (2 ml) was added and the reaction was agitated for 2.5 days at room temperature under Argon. The methyl-PEG-triarylphosphine conjugate was recovered by precipitation in diethyl ether (200 ml). The precipitate was redissolved in water, filtrated and lyophilized to afford the final product with 50% of subtitution. The product was used in the Staudinger reaction without purification. $^1$H NMR (400 MHz, $D_2O$): δ 3.37 (s, 3H, O-$CH_3$ (PEG)), 3.45-3.90 (m, 557 H, O-$CH_2$ (PEG)), 7.29–7.50 (m, 11H), 7.81–7.84 (m, 1H), 8.03–8.12 (m, 1H). $^{13}$C NMR (150 MHz, $CDCl_3$): δ 39.9, 40.1, 52.5, 59.3, 68.7, 69,5, 70.1, 70.3, 70.4, 70.6, 71.1, 72.0, 72.4, 128.5, 128.7, 128.8, 130.5, 131.0, 131.3, 132.0, 133.3, 134.1, 137.7, 138.1, 166.5, 166.7. $^{31}$P NMR (242 MHz, $CDCl_3$): δ –3.11. MALDI-TOF: 6034 D (calculated 6014 D).

**Conjugation.** To a solution of $TM_B$ in PBS pH 7.4 (300-500 nM, 100μl) was added a large excess (1 mg) of the lyophilized methyl-PEG-triarylphosphine conjugate. The reaction was heated at 37°C for 36 h to obtain maximum ligation. Reaction mixtures were divided in half and the two sets of reactions were subjected to SDS/PAGE in parallel. Proteins from the first gel were transferred to nitrocellulose membranes and visualized by Western blot analysis using a mouse monoclonal antibody to human thrombomodulin. This procedure monitored the presence of TM proteins that had reacted with the phosphine and were therefore PEG-labeled as well as unreacted TM. The second gel was stained with barium chloride/iodine solutions to reveal PEGylated molecules only (*21*).

# RESULTS AND DISCUSSION

It is now well established that the three consecutive EGF-like domains 4-6 (EGF4-6) of human TM exhibit full cofactor activity (*22*). The corresponding amino-acid sequence contains only one Methionine residue (Met-388), which is not vital for the protein bioactivity (*23,24*). According to the work of Kiick *et al.* (*19*) that used methionine analogs for bioconjugation, we surmised that the mutation of the Met-388 residue to Leucine (Leu) of a truncated TM fragment containing EGF4-6 and the insertion of a C-terminal Met might therefore be a good target for site-specific PEGylation.

To create a short recombinant bioactive TM mutant, we first used the DNA fragment encoding for the amino-acids sequence 349 to 492 to generate a gene containing a Met-388-Leu substitution and a C-terminal linker GlyGlyMet using site-directed mutagenesis (Figure 1). The final construct ($\mathbf{TM_{GGM}}$) was then inserted in the expression plasmid pET-39b(+). This plasmid contains a leader gene sequence coding for the *Dsba* enzyme, which is a periplasmic enzyme catalyzing the formation and isomerization of disulfide bonds of expressed proteins. We therefore expected the target TM mutant to possess all the disulfide bonds that natural human TM exhibit and that are required for proper protein folding and consequently cofactor activity. Expression under the induction of IPTG in *E. coli* methionine auxotroph (B834(DE3)/pET39-b/$TM_{GGM}$.) cultures depleted of methionine and supplemented with an azido-functionalized methionine analog: the azidohomoalanine (*19*) afforded the target protein as a N-terminal Dsba and Enterokinase enzymes fusion to a leader sequence containing hexahistine and S-tags. Protein expression was monitored by SDS/PAGE analysis and visualized by Western blot analysis. The target protein was not observed in the negative control culture, whereas TM fused to the leader sequence ($\mathbf{TM_A}$) was clearly detected in positive control cultures supplemented with azidohomoalanine. The accumulation of $\mathbf{TM_A}$ was taken as preliminary evidence for incorporation of the non-natural amino acid. $\mathbf{TM_A}$ was purified from the cell pellet by using immobilized metal-affinity chromatography with stepwise Imidazole gradient elution under native conditions. *Enterokinase* cleavage removed the fusion tag and generated the target proteins ($\mathbf{TM_B}$). N-terminal sequencing, amino acid compositional and mass analysis (Mass detected: 16545D) confirmed the integrity of $\mathbf{TM_B}$. On the western blot analysis of TM mutants (Figure 2), $\mathbf{TM_B}$ was characterized by a band around 33kD confirming that in some case $\mathbf{TM_B}$ proteins associate into dimers even under denaturing conditions as has been already reported (*25*).

Having expressed an azide-modified form of TM we next investigated the selective Staudinger ligation of this protein with a properly engineered methyl-PEG-triarylphosphine conjugate **2** (Scheme 1). We choose this ligation protocol in order to minimize the alteration of the TM mutant cofactor activity during the reaction. Indeed, in its classical form, the Staudinger reaction meets many of the criteria required of a chemoselective ligation in a cellular enviroment. The phosphine and the azide react rapidly and selectively in water at room temperature in high yield. Kiick *et al.* (*19*) prepared a triarylphosphine compound **1** that has been successfully used for the preparation of a protein-Flag conjugate as well as for labeling cell surface azide-bearing sialic acids with a biotinylated phosphine (*20*). In an identical manner, we used **1** to synthesize our methyl-PEG-triarylphosphine conjugate **2**. The carboxylic acid group of **1**

was reacted with the amino group of the commercially available derivative methyl-PEG-amine (m-PEG-$NH_2$ (5kD)) to create an amide bond (Scheme 1). The incorporation of the phosphino group to the PEG was checked by $^{1}H$, $^{13}C$ and $^{31}P$ NMR as well as MALDI-TOF spectroscopy (Figure 3). The peak at a mass of 6034 D observed for the methyl-PEG-triarylphosphine conjugate **2** is consistent with that expected for the increase of mass of 346.1 D brought by the linkage of the phosphino compound **1** to the initial m-PEG-$NH_2$ (Mass observed: 5668 D, Figure 3).

PEG polymers are well known to be highly hydrophilic. The addition of the hydrophilic m-PEG-$NH_2$ to **1** -totally hydrophobic- therefore dramatically enhanced the water solubility of the phosphino group in the bioconjugate **2**, which is crucial for the following Staudinger reaction. A large excess of lyophilized mehtyl-PEG-triarylphosphine conjugate **2** (1 mg) was added to 100 μL of an aqueous solution of $\mathbf{TM_B}$ in PBS at pH 7.4 and the Staudinger reaction was performed at 37 ℃ for 24h. The formation of the $\mathbf{TM_B}$-PEG conjugate was followed by western blotting analysis of the reaction mixture with time (Figure 2). The gel shows the apparition of a band at higher molecular weight than the initial $\mathbf{TM_B}$ with a simultaneous disappearance of the $\mathbf{TM_B}$ band. These results indicate that the Staudinger reaction proceeded efficiently in these conditions and were taken as primarily evidence for incorporation of the PEG polymer to the TM. As the reaction is not complete after 24h, we let it proceed for 12h more. Western blotting analysis (Figure 4a), and barium iodine staining (Figure 4b) of SDS-PAGE gels were then carried out in parallel to monitor the formation of $\mathbf{TM_B}$-PEG conjugate. On the western blot gel, the absence of the band characteristic of the starting $\mathbf{TM_B}$ revealed the bioconjugation of $\mathbf{TM_B}$ to PEG was 100 % complete. The SDS gel ran in parallel under identical conditions but stained with barium iodine confirmed the presence of PEG molecule in the bioconjugate by the presence of a band at similar molecular weight. The band showed under 20kd may be the unreacted mehtyl-PEG-triarylphosphine conjugate **2**. However, the mass of the PEGylated TM determined by SDS-PAGE analysis is different than that estimated based on the actual mass of the PEG attached. This variation in mass has been already reported (*26*) for the PEGylation of Hemoglobin. Indeed, studies of PEG in aqueous solution have shown that PEG typically binds 2-3 water molecules per ethylene oxide unit. Due to both the high flexibility of the backbone chain and the binding of water molecules, the PEG molecule exhibits an apparent size in the gel, which may not correspond to its actual molecular weight (*2*).

Activity assays were carried out to check the influence of the PEGylation on the TM cofactor activity (Table 1). We first investigate the activity of both $\mathbf{TM_A}$ and $\mathbf{TM_B}$ mutants and compared it to the one exhibited by the commercially recombinant human TM lacking the transmembrane domain. Indeed, Clarke et al. (*23*) has shown that the Met-388-Leu mutation results in a 2-fold increase in $k_{cat}$ for the activation of protein C by a thrombin-TM fragment complex. The alteration of the Met-388 to Leu in our TM mutants might therefore modify the bioactivity of the protein. However, within experimental errors, similar activity for commercially TM, $\mathbf{TM_A}$ and $\mathbf{TM_B}$ are observed. Our TM mutants therefore match the conformation and structure required for a proper thrombin binding and protein C activation. Moreover, the incorporation of PEG to $TM_B$ does not affect the cofactor activity of the protein, indicating that this site-specific PEGylation scheme does not interfere with thrombin binding and protein C activation.

# CONCLUSIONS

A thrombomodulin derivative containing EGF(4-6)-like domains designed with a azido-functionalized methionine C-terminal linker was successfully synthesized *via* a genetic engineering strategy. The protein exhibits fully bioactivity towards protein C. The TM construct was then conjugated to a novel engineered methyl-PEG-triarylphosphine compound *via* Staudinger reaction in mild conditions. Cofactor activity assay of both proteins before and after PEGylation were found to be similar indicating the success of the site-specific PEGylation scheme used. To our knowledge, we developed herein the first site-specific PEGylation at the C-terminal of a protein and therefore the first site-specific PEGylation of thrombomodulin-mimetic protein with full bioactivity.

# LEGENDS

**Scheme 1.** Synthesis of the methyl-PEG-triarylphosphine conjugate **2** and bioconjugation to the azido-functionalized $\mathbf{TM_B}$.

**Figure 1.** Structure of the TM mutant ($TM_{GGM}$).

**Figure 2.** Western blot analysis of TM mutants (4-20% SDS-PAGE gel): (1) purified $\mathbf{TM_A}$; (2) enterokinase cleavage of $\mathbf{TM_A}$ leading to the target protein $\mathbf{TM_B}$; (3-5) bioconjuation reaction with the PEG linker after 4h, 8h and 24h respectively. M: Molecular weight marker proteins.

**Figure 3.** MALDI-TOF Mass Spectrum of (a) the starting polymer m-PEG-$NH_2$ (mass detected (*m/z*): 5668 D) and (b) the methyl-PEG-triarylphosphine conjugate **2** (mass detected (*m/z*): 6034 D, calculated 6014 D).

**Figure 4.** (a) Western blot analysis and (b) barium iodine staining of a 10% SDS-PAGE gels: (1) and (3) bioconjuation reaction with the PEG linker; (2) initial azido-functionalized TM-derivative $\mathbf{TM_B}$. M: Molecular weight marker proteins.

**Table 1.** Dissociation constant and kinetic parameter determination for TM mutants.

# LITERATURE CITED

(1)Veronese, F. M. (2001) Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials 22*, 405-417.

(2)Roberts, M. J., Bentley, M. D., Harris, J. M. (2002) Chemistry for peptide and protein PEGylation. *Adv Drug delivery reviews 54*, 459-476.

(3)Katre, N.V. (1993) The conjugation of proteins with polyethylene glycol and other polymers. Altering properties of proteins to enhance their therapeutic potential. *Adv Drug Delivery Rev 10*, 91–114.

(4) Monkarsh, S.P., Ma, Y.M., Aglione, A., Bailon, P., Ciolek, D., DeBarbieri, B., Graves, MC., Hollfelder, K., Michel, H., Palleroni, A., Porter, J. E., Russoman, E., Roy, S., Pan, Y. C. E. (1997) Positional isomers of monopegylated interferon α2a: isolation, characterization, and biological activity. *Anal. Biochem. 247*, 434-440.

(5)Han, H-S., Yang, S-L., Yeh, H-Y., Lin, J-C., Wu, H-L., Shi, G-Y. (2001) Studies of a novel human thrombomodulin immobilized substrate: surface characterization and anticoagulation activity evaluation. *J. Biomater. Sci. Polymer edn, 12*, 1075-1089.

(6) Tsutsumi, Y., Onda, M., Nagata, S., Lee, B., Kreitman, R. J., Pastan, I. (2000) Site-specific chemical modification with polyethylene glycol of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) improves antitumor activity and reduces animal toxicity and immunogenicity. *Proc. Natl. Acad. Sci. USA 97*, 8548-8553.

(7) He, X.H., Shaw, P. C., Xu, L. H., Tam, S. C. (1999) Site-directed polyethylene glycol modification of trichosanthin: effects on its biological activities, pharmacokinetics, and antigenicity. *Life Sci. 64*, 1163-1175.

(8)Goodson, R.J., Katre, N.V. (1990) Site-directed pegylation of recombinant interleukin-2 at its glycosylation site. *Biotechnology 8*, 343–346.

(9)Kogan, T.P. (1992) The synthesis of substituted methoxy-poly(ethylene glycol) derivatives suitable for selective protein modification. *Synth. Commun. 22*, 2417–2424.

(10)Morpurgo, M., Veronese, F.M., Kachensky, D., Harris, J.M. (1996) Preparation and characterization of poly(ethylene glycol) vinyl sulfone. *Bioconjug. Chem. 7*, 363–368.

(11)Woghiren, C., Sharma, B., Stein, S. (1993) Protected thiol-polyethylene glycol: a new activated polymer for reversible protein modification. *Bioconjug. Chem. 4*, 314–318.

(12)Karpusas, M., Nolte, M., Benton, C.B., Meier, W., Lipscomb, W.N., Goelz, S. (1997) The crystal structure of human interferon beta at 2.2-A resolution. *Proc. Natl. Acad. Sci. USA 94*, 11813–11818.

(13) Kuan, C.T., Wang, Q. C., Pastan, I. (1994) *Pseudomonas* exotoxin A mutants. Replacement of surface-exposed residues in domain II with cysteine residues that can be modified with polyethylene glycol in a site-specific manner. *J. Biol. Chem. 269*, 7610-7616.

(14)Yamamoto, Y., Tsutsumi, Y., Yoshioka, Y., Nishibata, T., Kobayashi, K., Okamoto, T., Mukai, Y., Shimizu, T., Nakagawa, S., Nagata, S., Mayumi, T. (2003) Site-specific PEGylation of a lysine-deficient TNF-alpha with full bioactivity. *Nature Biotechnology 21*, 546-552.

(15)Weiler, H., Isermann, B. H. Thrombomodulin. (2003) *J. Thrombosis and Haemostasis 1*, 1515-1524.

(16) Kalafatis, M., Egan, J.O., Van't Veer, C., Cawthern, K.M., Mann, K.G. (1997) The regulation of clotting factors. *Crit. Rev. Eukaryot. Gene Expr. 7*, 241-280.

(17) Esmon, N.L., Owen, W.G., Esmon, C.T. (1982) Isolation of a membrane-boud cofactor for thrombin-catalyzed activation of protein-C. *J. Biol. Chem. 257*, 859-864.

(18)Esmon, C. T. (1992) The regulation of clotting factors. *Arteriosclevoris and Thrombosis 12*, 135-145.

(19)Kiick, K. L., Saxon, E., Tirrell, D. A., Bertozzi, C. R. (2002) Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. *PNAS 99*, 19-24.

(20)Saxon, E., Bertozzi, C. R. (2000) Cell surface engineering by a modified Staudinger reaction. *Science 287*, 2007-2010.

(21) Kurfurst, M. M. (1992) Detection and molecular weight determination of polyethylene glyol-modified hirudin by staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem. 200*, 244-248.

(22)Parkinson, J. F., Nagashima, M. M, Kuhn, I., Leonard, J., Morser, J. (1992) Structure-Function studies of the epidermal growth-factor domains of human thrombomodulin. *Biochem Biophys. Res. Commun. 185*, 567-576.

(23)Clarke, J. H., Light, D. R., Blasko, E. J., Parkinson, F., Nagashima, M., McLean, K., Vilander, L., Andrews, W. H., Morser, J., Glaser, C. B. (1993) The short loop between epidermal growth factor-like domain-4 and domain-5 is critical for human thrombomodulin function. *J. Biol. Chem. 268*, 6309-6315.

(24)Nagashima, M., Lundh, E., Leonard, J. C., Morser, J., Parkinson J. F. (1993) Alanine-scanning mutagenesis of the epidermal growth factor-like domains of human thrombomodulin identifies critical residues for its cofactor activity. *J. Biol. Chem. 268*, 2888-2892.

(25) Glaser, C. B., Morser, J.,Clarke, J. H., Blasko, E., McLean, K., Kuhn, I., Chang, R. –J., Lin, J.-H., Vilander, L., Andrews, W. H., and Light, D. R. (1992) Oxidation of a secific methionine in thrombomodulin by activated neutrophil products blocks cofactor activity-a potential rapid mechanism for modulation of coagulation. *Clin. Invest. 90*, 2565-2573.

(26)Manjula, B. N., Tsai, A., Upadhya, R., Perumalsamy, K., Smith, P. K., Malavalli, A., Vandegriff, K., Winslow, R. M., Intaglietta, M., Prabhakaran, M., Friedman, J. M., Acharya, A. S. (2003) Site-specific PEGylation of hemoglobin at cys-93(beta): Correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain. *Bioconjugate Chemistry 14*, 464-472.

# Site-Specific PEGylation of a Thrombomodulin Derivative

**Chrystelle S. Cazalis, Carolyn A. Haller, and Elliot L. Chaikof**

*Department of Surgery, Emory University, 1639 Pierce Drive, room 5105, Atlanta, GA 30322.*

## INTRODUCTION

Pegylation of proteins increases both their molecular size and steric hindrance, which results in an improvement of protein plasma half-life and resistance to proteolytic cleavage. In addition, protein immunogenicity may be decreased[1,2]. Characteristically, PEGylation usually involves the reaction to available lysine amino groups, some of which may be within or near a bioactive site. Thus, most protocols are nonspecific and result in a loss of protein activity. For example, Han *et al.*[3] conjugated human thrombomodulin (TM) to PEG via trichlorotriazine as the coupling agent for immobilization onto a glass surface. Despite successful immobilization, they reported reduced TM activity presumably due to the alteration of protein conformation after PEGylation. To overcome this problem, several approaches have been proposed. Site-specific PEGylation can be achieved through the introduction of a cysteine residue in engineered proteins with a free thiol available for conjugation reactions[4,5,6,7,8,9,10]. The efficacy of this approach is compromised, however, by a low yield of PEGylated proteins and often by a substantial loss of activity due to the introduction of the cysteine residue[11]. As an alternative strategy, Yamamoto *et al.*[12] reported the site-specific PEGylation of a lysine-deficient tumor necrosis factor (TNF)-α at its N-terminus using an activated PEG (methoxy-PEG-succinimidyl propionate). The corresponding bioconjugate showed higher bioactivity *in vitro* and greater antitumor therapeutic potency than randomly mono-PEGylated TNF-α.

We report herein the use of a similar strategy for site-specific PEGylation of human thrombomodulin . This transmembrane protein is a critical regulator of the protein C pathway and represents a major anticoagulant mechanism that is operative under physiologic conditions in vivo[13,14]. TM is a cofactor for thrombin-catalyzed activation of protein C, enhancing the rate of the reaction by 1000-fold[15]. Using genetically-directed synthesis in *Escherichia coli*, we expressed a short TM construct containing EGF-like domains 4-6 and an azido-functionalized methionine analog[16] as a C-terminal linker. PEGylation was then achieved through Staudinger ligation.[17]

## EXPERIMENTAL

**Materials.** All chemical reagents were obtained from Sigma Chemical Corporation (St. Louis, MS). The BamH I and Shrimp Alkaline Phosphatase enzymes were obtained from New England Biolabs, Inc. (Beverly, MA). The Quikchange Site-Directed Mutagenesis kit was from Stratagene (La Jolla, CA). E.coli strain B834 (DE3), plasmid pET-39b(+), S-Tag Rapid Assay kit and Site-Specific Enterokinase Cleavage and Capture Kits were from Novagen (Madison, WI). All plasmid purification kits were purchased from QIAGEN Inc. (Chatsworth, CA). The mouse monoclonal antibody to human thrombomodulin was from COVANCE Corp. (Richmond, CA). Synthetic oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). All reagents for manipulating DNA and bacteria were sterilized by autoclave.

**Synthesis of the methyl-PEG-triarylphosphine conjugate 2.** A mixture of the phosphino reagent **1** (10.3 mg, 28 μmol) and 1,3-dicyclohexylcarbodiimide (DCC) (5.7 mg, 27 μmol) in $CHCl_3$ (2 ml) was agitated for 1h at room temperature under Argon. A solution of the methyl-PEG-amine derivative (m-PEG-$NH_2$ ($M_n$ 5kD)) (120 mg, 24 μmol) in $CHCl_3$ (2 ml) was then added and the reaction agitated for 2.5 days at room temperature under Argon. The methyl-PEG-triarylphosphine conjugate was recovered by precipitation in diethyl ether (200 mL). The precipitate was then redissolved in water, filtrated, and lyophilized to afford the final product with 50% of substitution. The product was used in the Staudinger reaction without further purification. $^1$H NMR (400 MHz, $D_2O$): δ 3.37 (s, 3H, O-$CH_3$ (PEG)), 3.45-3.90 (m, 557 H, O-$CH_2$ (PEG)), 7.29–7.50 (m, 11H), 7.81–7.84 (m, 1H), 8.03–8.12 (m, 1H). $^{13}$C NMR (150 MHz, $CDCl_3$): δ 39.9, 40.0, 52.5, 59.3, 68.7, 69,5, 70.1, 70.3, 70.4, 70.6, 71.1, 72.0, 72.4, 128.5, 128.7, 128.8, 130.5, 131.0, 131.3, 132.0, 133.3, 134.1, 137.7, 138.1, 166.5, 166.7. $^{31}$P NMR (242 MHz, $CDCl_3$): δ –3.11. MALDI-TOF: 6034 D (calculated 6014 D).

## RESULTS AND DISCUSSION

**Synthetic gene construction.** A DNA fragment encoding for EGF (4-6) domains of human TM was obtained by polymerase chain reaction using primers 5'-TACCCTAACTACGACCTGGTG-3' and 5'-TATGAGCAAGCCCGAATG-3'. Through a series of intermediate constructs, this fragment was used to generate a gene containing a Leucine (Leu) substitution for Methionine-388 (Met-388), N-terminal and C-terminal BamH I sites and a C-terminal linker GlyGlyMet using site-directed mutagenesis. The final construct ($TM_{GGM}$) was then inserted using the BamH I site of the expression plasmid pET-39b(+). All mutations were verified through sequence analysis. The alteration of the Met-388 to Leu (Figure 1) was performed in order to increase the bioactivity of the TM-derivatives. Indeed, White et al.[18] has shown that the Met-388-Leu mutation results in a 2-fold increase in $k_{cat}$ for the activation of protein C.

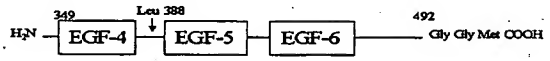
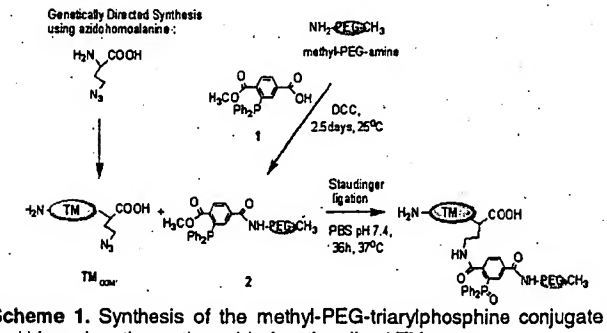



**Figure 1.** Structure of the TM-mimetic protein.

**Protein expression and purification.** pET-39b(+)/$TM_{GGM}$ was introduced into the E. coli methionine auxotroph strain B834(DE3). Expression under the induction of 1 mM IPTG in M9 medium supplemented with 100 mg/L of methionine analog[16,17] afforded the target protein as a N-terminal Dsba fusion protein containing a hexahistidine tag for purification and a S-tag for quantification. Dsba is a periplasmic enzyme that catalyses the formation and isomerization of disulfide bonds in pET-39b. Electrophoretic analysis of the whole cell lysate as a function of time via western blot analysis using a mouse monoclonal antibody to human TM indicated the gradual accumulation of new protein during a 4.5 hr induction period. The molar concentration of the target protein in the cell pellet was detected with the S-tag rapid assay. The yield of protein was approximately 17 mg per liter of cell culture. Enterokinase cleavage removed the fusion tag and generated the target protein ($TM_{GGM}$). N-terminal sequencing, amino acid compositional and mass analysis confirmed the integrity of $TM_{GGM}$ (Mass detected (m/z): 16 545.2 D (calculated 16 540.0 D)).

**TM-PEG conjugation.** The methyl-PEG-triarylphosphine conjugate **2** was prepared from a commercially available methyl-PEG-amine derivative (m-PEG-$NH_2$ ($M_n$ 5kD)) and the phosphino reagent **1**[16] (Scheme 1).




**Scheme 1.** Synthesis of the methyl-PEG-triarylphosphine conjugate **2** and bioconjugation to the azido-functionalized $TM_{GGM}$.

An excess (1 mg) of the lyophilized methyl-PEG-triarylphosphine conjugate **2** was added to a solution of $TM_{GGM}$ in PBS pH 7.4 (300-500

nM, 100μL). The reaction was then heated at 37°C for 36 h. The reaction mixture was subjected to SDS/PAGE and visualized by Western blot analysis in order to monitor PEGylation of $TM_{GGM}$. As noted in Figure 2 (lane 2), $TM_{GGM}$ is characterized by a band at around 31-33kD that corresponds to a TM dimer. Indeed, thrombomodulin often self-associates as dimers even under denaturing conditions[19]. The presence of a band at 38-42 kD (Figure 2, lane1) with disappearance of the initial $TM_{GGM}$ band is consistent with the formation of a PEG-TM conjugate. In addition, the approximately 10 kD shift indicates that each TM monomer has conjugated to a 5kD PEG molecule. These results indicate that the Staudinger reaction proceeded efficiently in these conditions and were taken as primarily evidence for conjugation of the PEG polymer to $TM_{GGM}$.

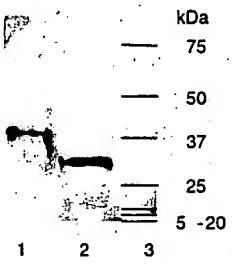



**Figure 2.** Western blot analysis of TM-derivatives. Lane 1. Bioconjugation reaction with the PEG linker. Lane 2. $TM_{GGM}$. Lane 3. Molecular weight marker proteins.

Measured catalytic activity for commercially available human TM ($k_{cat}/K_M$ = 0.21 ± 0.05 $min^{-1}$. $\mu M^{-1}$) and $TM_{GGM}$ ($k_{cat}/K_M$ = 0.20 ± 0.05 $min^{-1}$. $\mu M^{-1}$) were similar. Moreover, the conjugation of PEG to $TM_{GGM}$ did not affect activity ($k_{cat}/K_M$ = 0.16 ± 0.05 $min^{-1}$. $\mu M^{-1}$). These data confirmed that this site-specific PEGylation scheme does not interfere with thrombin binding and protein C activation.

## CONCLUSIONS

A thrombomodulin derivative containing EGF(4-6)-like domains with an azido-functionalized methionine C-terminal linker was successfully synthesized via a genetic engineering strategy. The TM mutant was site-specifically conjugated at the C-terminus to methyl-PEG-triarylphosphine *via* Staudinger reaction in mild conditions. Enzyme activity before and after PEGylation were similar indicating the utility of this site-specific PEGylation scheme.

## REFERENCES

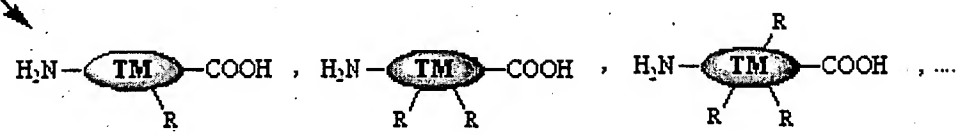
**References**

1. Veronese, F.M. *Biomaterials* **2001**, *22*, 405.
2. Roberts, M.J.; Bentley, M.D.; Harris, J.M. *Adv Drug Delivery Reviews* **2002**, *54*, 459.
3. Han, H.S.; Yang, S.L.; Yeh, H.Y.; Lin, J.C.; Wu, H.L.; Shi, G.Y. *J. Biomater. Sci. Polymer edn* **2001**, *12*, 1075.
4. Tsutsumi, Y.; Onda, M.; Nagata, S.; Lee, B.; Kreitman, R.J.; Pastan, I. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 8548.
5. He, X.H.; Shaw, P.C.; Xu, L.H.; Tam, S.C. *Life Sci.* **1999**, *64*, 1163.
6. Goodson, R.J.; Katre, N.V. *Biotechnology* **1990**, *8*, 343.
7. Kogan, T.P. *Synth. Commun.* **1992**, *22*, 2417.
8. Morpurgo, M.; Veronese, F.M.; Kachensky, D.; Harris, J.M. *Bioconjug. Chem.* **1996**, *7*, 363.
9. Woghiren, C.; Sharma, B.; Stein, S. *Bioconjug. Chem.* **1993**, *4*, 314.
10. Karpusas, M.; Nolte, M.; Benton, C.B.; Meier, W.; Lipscomb, W.N.; Goelz, S. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 11813.
11. Kuan, C.T.; Wang, Q.C.; Pastan, I. *J. Biol. Chem.* **1994**, *269*, 7610.
12. Yamamoto, Y.; Tsutsumi, Y.; Yoshioka, Y.; Nishibata, T.; Kobayashi, K.; Okamoto, T.; Mukai, Y.; Shimizu, T.; Nakagawa, S.; Nagata, S.; Mayumi, T. *Nature Biotechnology* **2003**, *21*, 546.
13. Weiler, H.; Isermann, B. H. *J. Thrombosis and Haemostasis* **2003**, *1*, 1515.
14. Kalafatis, M.; Egan, J.O.; Van't Veer, C.; Cawthern, K.M.; Mann, K.G. *Crit. Rev. Eukaryot. Gene Expr.* **1997**, *7*, 241.
15. Esmon, N.L.; Owen, W.G.; Esmon, C.T. *J. Biol. Chem.* **1982**, *257*, 859.
16. Kiick, K. L.; Saxon, E.; Tirrell, D. A.; Bertozzi, C. R. *PNAS* **2002**, *99*, 19.
17. Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007.
18. White, C.E.; Hunter, M.J.; Meininger, D.P.; White, L.R.; Komives, E.A. *Protein Engineering* **1995**, *3*, 1177.
19. Glaser, C.B.; Morser, J.; Clarke, J.H.; Blasko, E.; McLean, K.; Kuhn, I.; Chang, R.J.; Lin, J.H.; Vilander, L.; Andrews, W.H.; Light, D.R.; *J. Clin. Invest.* **1992**, *90*, 2565.

bioactivity. Typically polyethylene glycol ("PEG") is used as the polymer spacer. An **advantage** of PEGylation of proteins is an increase in plasma half-life, stability against proteolytic cleavage, and a decrease of protein immunogenicity.

**iii. Attach sketches, drawings, photographs, or other material that help illustrate the description, if appropriate.**

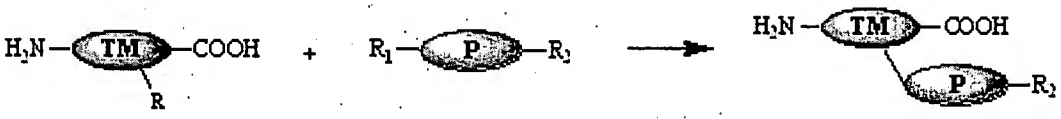
**Genetically Directed Synthesis using non-natural amino-acid :**




TM analogs containing NNAA

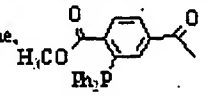
TM: Thrombomodulin analog capable of activating protein C
$NH_2$: Amino group at the N-terminal of TM
COOH: Carboxylic acid group C-terminal of TM
NNAA: Non-Natural Amino Acid
R: Functional group of the non-natural amino acid ( $N_3$, alkyl, diene, ....)

**Example of a Conjugation Reaction:**




TM analog containing one NNAA  Linear polymer  TM conjugate

P: Linear or branched natural or synthetic polymers such as PEG, oligosaccharides, ...

$R_1$ : Alkyne, diene,  , ....

$R_2$ : - Functional group for anchoring onto surface : alkyne, diene, biotin, ....

- Anti-inflammatory/anti-thrombotic groups such as heparin, sialic acid Lewis X, ....

Scheme 1.

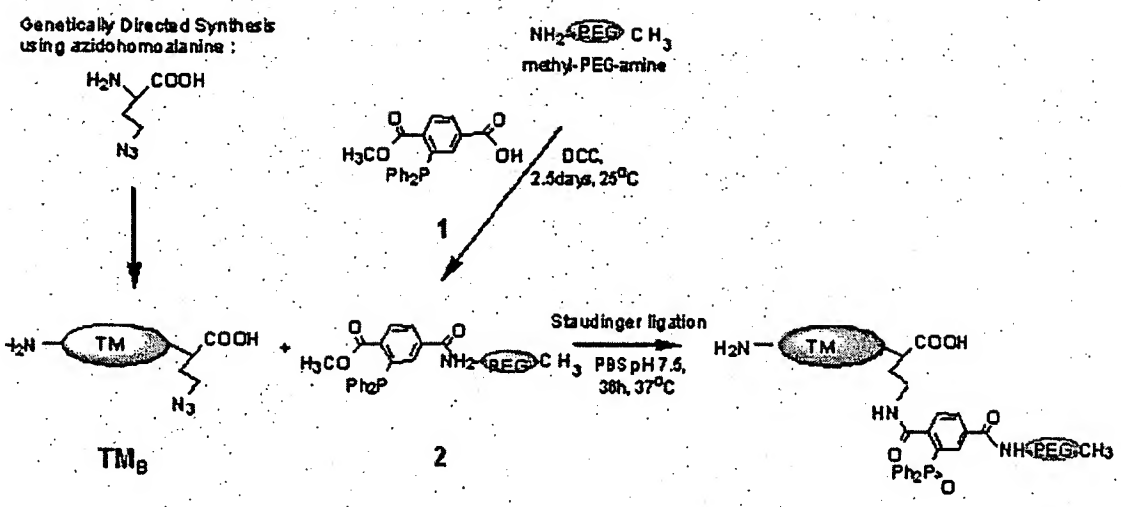

Genetically Directed Synthesis using azidohomoalanine :
methyl-PEG-amine
DCC, 2.5days, 25°C
1
Staudinger ligation
PBS pH 7.5, 36h, 37°C
TM
COOH
TMB
2

**Figure 1.**

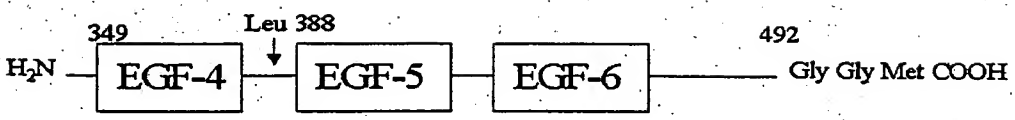


Figure 2.

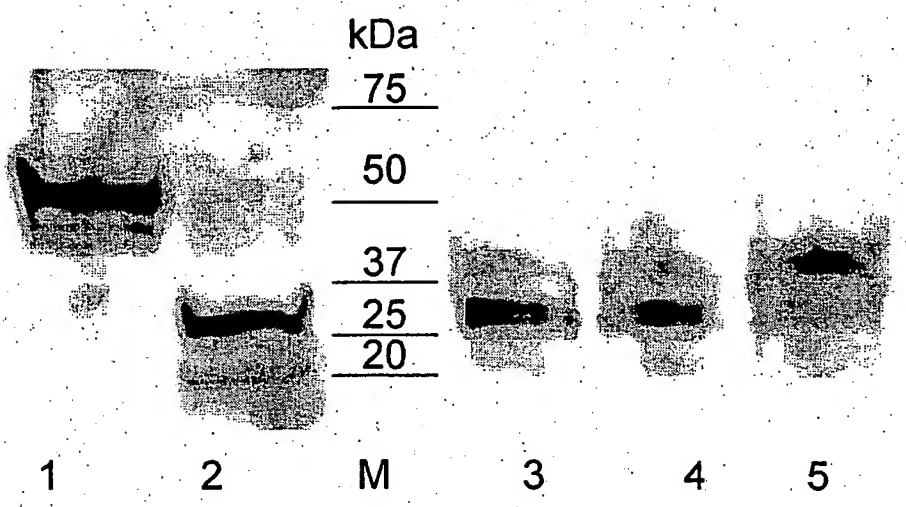


Figure 3.

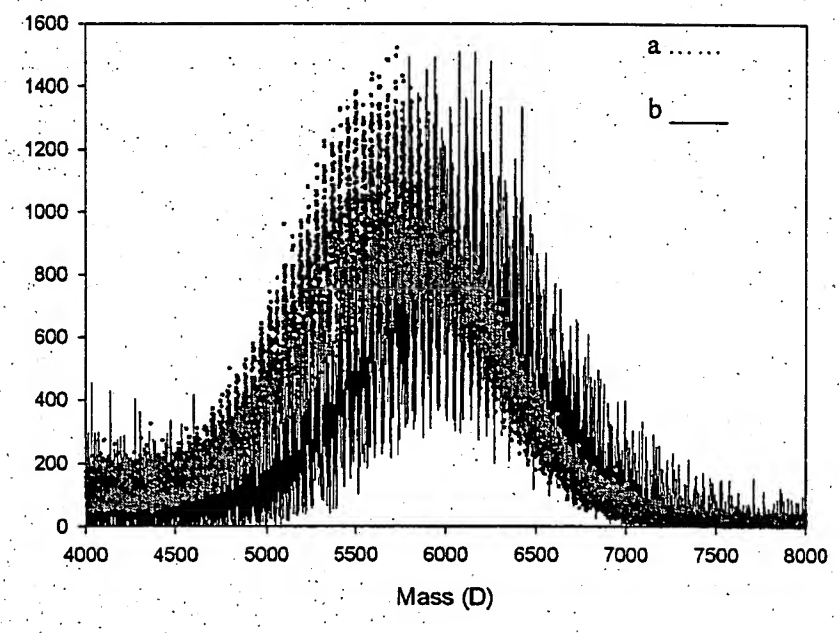


**Figure 4.**

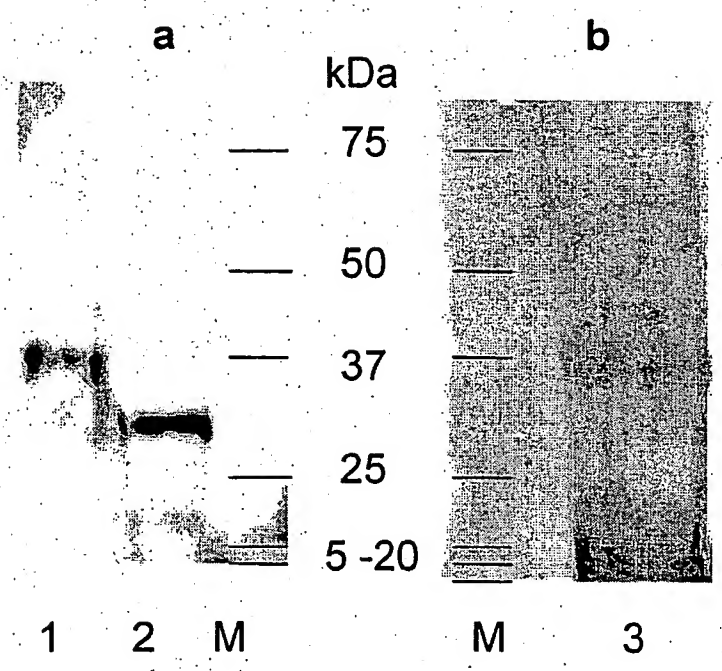


**Table 1.** Kinetic Parameters For TM Mutants

| | $TM_A$ | $TM_B$ | PEGylated $TM_B$ | Commercial TM |
|---|---|---|---|---|
| $K_M$ (mM) | 0.9 ± 0.2 | 1± 0.5 | 1 ± 0.5 | 0.7 ± 0.1 |
| $k_{cat}$ ($min^{-1}$) | 0.22 ± 0.05 | 0.16 ± 0.05 | 0.20 ± 0.05 | 0.14 ± 0.02 |
| $k_{cat}/K_M$ ($min^{-1}$. $mM^{-1}$) | 0.26 ± 0.1 | 0.16 ± 0.05 | 0.20 ± 0.05 | 0.21 ± 0.05 |

# Generation of Thrombomodulin Conjugates and Applications Thereof

Elliot L. Chaikof, M.D., Ph.D., Chrystelle S. Cazalis, Ph.D, Carolyn A. Haller, Ph.D.
Emory University
404-727-8413
echaiko@emory.edu

## Patent Claims:

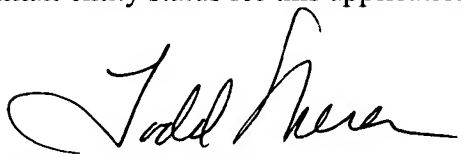
1. Generation of recombinant thrombomodulin constructs containing non-natural amino acids
   - A preferred embodiment of the invention include the expression of the extracellular portion of thrombomodulin
   - A preferred embodiment of the invention include the expression of the extracellular portion of thrombomodulin containing catalytically active sites
   - A preferred embodiment of the invention include the expression of the extracellular portion of thrombomodulin that is capable of activating protein C
   - A preferred embodiment of the invention include the expression of thrombomodulin mutants that contain single or multiple non-natural amino acids
   - Examples of non-natural amino acids include: methionine analogues, alanine analogues, phenylalanine analogues, leucine analogues, proline analogues and isoleucine analogues. Example of methionine analogues include: L-2-amino-4-azido-butanoic acid.
   - A preferred embodiment of the invention include the expression of thrombomodulin mutants containing a single non-natural amino acid at the C-terminal portion of the construct
2. Generation of recombinant thrombomodulin constructs that are conjugated to natural or synthetic polymers via non-natural amino acids in the recombinant protein
   - An embodiment of the invention include the conjugation of the construct to synthetic polymer or natural polymers
   - Examples of linear or branched synthetic polymers for conjugation include: poly(t-butyl acrylate), poly(t-butyl methacrylate), polyacrylamide, glycolipid and their mimetics.
   - Example of natural polymers include: glycoproteins and their mimetics, poly(arginine), polysaccharides and their mimetics.
   - An embodiment of the invention include the conjugation of the construct to linear or branched poly(ethylele glycol)
   - A preferred embodiment of the invention include the conjugation of the construct to linear poly(ethylele glycol)
3. Generation of recombinant thrombomodulin constructs or derivatives thereof that are conjugated to multifunctional natural or synthetic polymers for surface anchoring
   - An embodiment of the invention includes the conjugation of the construct or derivatives thereof to synthetic polymers for surface anchoring of the conjugate
   - A preferred embodiment of the invention include the conjugation of the construct to poly(ethylene glycol) for surface anchoring of the conjugate

- Examples of anchoring groups include: biotin, conjugated diene, azide, alkyne, diphenylphosphine, triarylphosphine.
- Examples of surface targeting groups include: sialyl-Lewis X, antibodies or Fab fragments agains VCAM-1, ICAM-1 or other inflammatory cell surface proteins, antifibrin antibody.
- An embodiment of the invention include the conjugation of the construct to synthetic polymers for anchoring to the surfaces of synthetic or natural materials
- Examples of synthetic materials include: poly(tetrafluoroethylene) , polysiloxanes, poly(ether urethane urea), poly(lactic acid-co-glycolic acid), glass surface and derivatives.
- Examples of natural materials include: cells, tissues, blood vessels
- An embodiment of the invention consists of coating of the surface of medically implanted or human tissue or fluid contacting devices including but not restricted to vascular grafts, stents, heart valves, dialysis membranes, membrane oxygenators, catheters, or guide wires to alter surface properties.
- An embodiment of the invention consists of coating of the surface of living cells or tissues, including, but not restricted to smooth muscle cells, fibroblasts, endothelial cells, stem cells, chondrocytes, osteoblasts, pancreatic islets, or genetically engineered cells.

4. Generation of recombinant thrombomodulin constructs or derivatives thereof that are conjugated to multifunctional natural or synthetic polymers that contain other anti-inflammatory or anti-thrombotic properties
   - An embodiment of the invention includes the conjugation of the construct or derivatives thereof to synthetic polymers that contain one or more additional anti-inflammatory groups
   - Examples of anti-inflammatory groups include: sialic acids and their mimetics/derivatives
   - An embodiment of the invention includes the conjugation of the construct or derivatives thereof to synthetic polymers that contain one or more additional anti-coagulant/anti-thrombotic groups
   - Examples of anti-coagulant/anti-thrombotic groups include: heparin and its mimetics/derivatives
   - An embodiment of the invention includes its use as a systemic agent for treatment of micro or macrovascular blood clots, stroke, heart attack, disseminated intravascular coagulation or other inflammatory or prothrombotic condition.

**SMALL ENTITY STATEMENT**

In accordance with 37 C.F.R. 1.27, I assert that Emory University is entitled to small entity status for this application, Emory docket number 04027Prov.



Todd T. Sherer, Ph.D.
Registration No. 39,369
January 20, 2004